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Waterborne and Surface-Associated Carbohydrates as Settlement Cues for Larvae of the Specialist Marine Herbivore *Alderia modesta*

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Abstract. Larvae of the specialist marine herbivore *Alderia modesta* (Opisthobranchia: Ascoglossa) metamorphose in response to a chemical settlement cue from the alga *Vaucheria longicaulis*, the obligate adult prey. Bioactivity coeluted with both high and low molecular weight carbohydrates in solution, and with insoluble high molecular weight carbohydrates associated with the algal cell wall. Larvae metamorphosed in response to water conditioned by *V. longicaulis*, as well as to frozen and homogenized algal tissue. The inducer was efficiently extracted from the algae with boiling water, but after all soluble activity was extracted, residual tissue still induced larval settlement. Ethanol precipitation of a boiled-water extract followed by gel filtration chromatography showed that the precipitate contained carbohydrates of >100,000 Da molecular weight, while the supernatant contained only low molecular weight carbohydrates (<2,000 Da); in both cases all activity was associated with the carbohydrate peak. An aqueous-insoluble 4% NaOH extract was chromatographed in 7 M urea to yield a bioactive high molecular weight carbohydrate peak. Activity was not affected by proteinase K or mild acid hydrolysis, but was significantly decreased by periodate treatment. The results indicate that larvae of *A. modesta* metamorphose in response to both water-soluble and surface-associated carbohydrates of *V. longicaulis*, and that the soluble cue exists as both high and low molecular weight isoforms.

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Abbreviations: BVE = boiled *Vaucheria* extract.

Introduction

Most marine invertebrate species produce free-swimming larvae that disperse in the plankton until becoming competent to settle to the bottom and metamorphose into the adult form (Grahame and Branch, 1985; Levin and Bridges, 1995). Larval recruitment plays a critical role in benthic marine ecosystems, structuring communities and regulating population dynamics (Grosberg, 1982; Roughgarden *et al.*, 1988; Underwood and Fairweather, 1989). Microscopic larvae are generally viewed as passive particles transported by flow to the benthos (Eckman, 1983, 1990; Butman, 1987). Following hydrodynamic delivery of larvae to the bottom, recruitment can be divided into settlement and metamorphosis (Chia and Koss, 1988; Pawlik, 1992). Settlement is characterized by active behaviors with which larvae explore the physical and chemical characteristics of potential substrata (LeTourneux and Bourget, 1988; Rodriguez *et al.*, 1993). Larvae may reject a substrate and resume swimming, becoming resuspended in the water column (Butman *et al.*, 1988; Butman and Grassle, 1992). Alternatively, larvae may respond to surface-associated cues and commit to metamorphosis, an irreversible developmental transformation into the adult stage of the organism (Burke, 1983; Pawlik *et al.*, 1991; Roberts *et al.*, 1991; Pawlik, 1992). Larvae are capable of fine-scale discrimination among substrata both in the laboratory and in the field (Keough and Downes, 1982; Raimondi, 1988).

Recent studies have demonstrated that both surface-associated and water-soluble chemical cues can trigger larval behavioral responses that greatly increase rates of settlement and metamorphosis. Still-water laboratory assays have demonstrated the importance of surface-associated chemical cues for inducing larval metamorphosis of barnacles (Maki

et al., 1990), bryozoans (Hurlbut, 1991), corals (Morse *et al.*, 1988), gastropods (Morse *et al.*, 1984), and polychaetes (Kirchman *et al.*, 1982). Hydrodynamic conditions and the presence of a surface cue associated with adult conspecifics had an interactive effect on settlement of larvae of the reef-building polychaete *Phragmatopoma californica* in flow (Pawlik *et al.*, 1991). Waterborne chemical cues also affect larval settlement processes. Soluble cues secreted by the adult prey organisms induced settlement and metamorphosis in the opisthobranchs *Phestilla sibogae* and *Adalaria proxima* (Hadfield and Scheuer, 1985; Lambert and Todd, 1994). Larvae of the oyster *Crassostrea virginica* showed dramatic behavioral responses to a chemical cue secreted by adult conspecifics, increasing settlement in both still and moving water (Tamburri *et al.*, 1992; Turner *et al.*, 1994; Tamburri *et al.*, 1996). However, despite decades of research into the nature of larval chemical settlement cues, relatively little is known about the molecules that regulate this crucial aspect of the life history of most benthic marine invertebrates.

A recent study of a population of the opisthobranch mollusc *Alderia modesta* revealed several unusual features that make *A. modesta* an ideal experimental system for investigating larval life history and settlement processes (Krug, 1998a, b). *A. modesta* is an ascoglossan found in temperate estuaries in association with its obligate food source, the yellow-green alga *Vaucheria longicaulis* (Xanthophyta: Xanthophyceae) (Hartog and Swennen, 1952; Hartog, 1959; Trowbridge, 1993). In southern California, *A. modesta* exhibits a reproductive polymorphism that is extremely rare among marine invertebrates; study populations contain specimens that produce planktotrophic larvae and other individuals that produce lecithotrophic larvae (Krug, 1998b). Most lecithotrophic spawn masses contain a mixture of sibling larvae, some of which metamorphose spontaneously within 2 days of hatching; the remaining veligers delay metamorphosis until encountering a chemical cue derived exclusively from the adult host alga *V. longicaulis* (Krug, 1998a). The present work used a bioassay for larval metamorphosis to determine whether the inductive activity was soluble or surface-associated in nature, and for bioassay-guided isolation of active fractions as a preliminary step in purifying the settlement cue.

Materials and Methods

Collection of organisms and larval bioassay

Alderia modesta (Lovén, 1844) and *Vaucheria longicaulis* were collected from mudflats in the Kendall-Frost Marine Reserve and Northern Wildlife Preserve, and in the San Diego River Flood Control Channel, San Diego, California, U.S.A. All algae used in this study conformed to published descriptions of *V. longicaulis* from California (Abbott and Hollenberg, 1976). Patches of *V. longicaulis* were grown under continuous lighting in the laboratory, and blades of algae were pulled free of the sediment base and rinsed in seawater before use in assays. Adult specimens of *A. modesta* were maintained in petri dishes under 1 cm of seawater, and lecithotrophic egg masses were harvested daily for 3 days. Egg masses from each day were pooled and maintained in 0.45 μm-filtered seawater (FSW); water was changed every other day until hatching. Upon hatching, larvae were maintained in FSW for 2 days, to allow spontaneous metamorphosis to occur in cue-independent larvae (Krug, 1998a). The remaining larvae were then subsampled for use in the bioassay. For each experimental treatment, 15 larvae were added to each of 3 replicate dishes containing 4 ml FSW. After 2 days, larvae were scored for metamorphosis. Each experiment included a FSW-only treatment as a negative control and live *V. longicaulis* as a positive control. The percentage of metamorphosis for each replicate was arcsine transformed, and treatments were compared using a 1-way ANOVA. Unplanned comparisons of means were done using the Scheffé procedure (Day and Quinn, 1989).

Secretion of settlement cue

An experiment was designed to determine whether the *Vaucheria*-derived settlement cue was surface-associated or secreted by the algae. Small patches (1 cm^2) of *V. longicaulis* were cut from a growing mat and left attached to the sediment base. Conditioned seawater (CSW) was made by placing a patch in 4 ml FSW for either 3 h or 24 h, after which the CSW was filtered through cotton and placed in a sterile petri dish; larvae were added directly to the CSW for the bioassay. Conditioned fresh water (CFW) was made by placing patches of *V. longicaulis* in 4 ml deionized water for 24 h. The CFW was filtered through cotton, dried on a rotary evaporator, and resuspended in an equivalent volume of FSW for use in the bioassay. The negative control was FSW aged 24 h and filtered through cotton in parallel with treatements; the positive control was live *V. longicaulis* tissue.

To determine whether *Vaucheria longicaulis* must be alive to trigger metamorphosis, pieces of the algae were frozen at $-20°C$ for 3 days. Frozen patches were thawed by immersion in FSW at room temperature for 1 h prior to use in the bioassay. To determine whether algal tissue must be physically intact, blades of live *V. longicaulis* were pulled free of a 2 cm^2 sediment base and washed in FSW. The algae was manually homogenized in 10 ml deionized water for 20 min, and the suspension sonicated for another 10 min. The homogenate was centrifuged (10 min, 2000 RPM) and the supernatant removed. The soluble homogenate was assayed by adding 200 μl (high concentration) or 30 μl (low concentration) aliquots to 4 ml FSW for use in the bioassay. The negative control was FSW, and the positive control was live intact *V. longicaulis* tissue.

Sequential extraction with boiling water

Four 20 × 20 cm mats of *Vaucheria longicaulis* attached to the natural sediment base were field collected (March 1997) and grown in the laboratory under continuous lighting, moistened daily with 50% seawater. After 2 weeks algal blades had grown 1–2 cm in height, and were harvested by cutting with dissecting scissors just above the sediment base. The *V. longicaulis* tissue (1.34 g wet weight) was placed in a beaker containing 50 ml deionized water and boiled for 10 min. The solution of boiled *Vaucheria* extract (BVE) was filtered through 100 μm Nitex mesh to remove *Vaucheria* residue, and then through a 0.45 μm filter membrane. The *Vaucheria* residue was collected off of the mesh filter, put in 50 ml of fresh deionized water, and again boiled for 10 min to generate a second extract. This process was repeated four more times, yielding a total of six sequential boiling water extracts. The *Vaucheria* residue remaining after the sixth extraction was collected from the filter; this residue was yellow-brown in coloration but the blades were still physically intact. Each of the six extracts was assayed by adding a 50 μl aliquot to 4 ml FSW per replicate assay dish. Pieces of live *V. longicaulis* were assayed as a positive control, and equivalently sized pieces of the *V. longicaulis* residue remaining after the six sequential extractions were also assayed.

Biochemical characterization of boiled Vaucheria longicaulis *extract (BVE)*

The initial extract made by boiling *Vaucheria longicaulis* for 10 min (described above) was subjected to preliminary biochemical characterization. Six volumes of ethanol were added to 1 ml of BVE and the solution was precipitated overnight at 4°C. The precipitate was pelleted by centrifugation, the supernatant removed, and the precipitate washed with ethanol and repelleted. The supernatant and wash ethanol were combined and dried on a rotary evaporator. The precipitate and supernatant residue were individually resuspended in 1 ml of MilliQ-purified water, such that the material in each fraction was present in solution at the same concentration as in the original extract.

Aliquots (100 μl) of the initial BVE and of the resuspended solutions of supernatant and precipitate were used in subsequent assays to determine the dry weight, carbohydrate content, protein content, and bioactivity of each sample. Lyophilized aliquots were weighed to determine dry mass. Carbohydrate content was determined for duplicate aliquots from each sample using the phenol-sulphuric colorimetric assay (DuBois *et al.*, 1956). Measurements were calibrated to a standard curve generated with known concentrations of glucose. Protein content was determined using the BCA colorimetric assay (Pierce Co.) calibrated to a standard curve generated with commercially supplied albumin standards. Bioactivity was determined using the larval settlement bioassay.

Another 3 ml of BVE was precipitated with 6 volumes of ethanol overnight, and the supernatant and precipitated material were separated as before. The carbohydrate elution profiles of both the supernatant and precipitate fractions were determined using a gel filtration column (90 cm × 1 cm) of Sephacryl S-200 resin (Pharmacia Co.). The column was calibrated for molecular weight using Blue Dextran to determine the void volume (V_o) and glucose to determine the included volume (V_i) for small molecules; size standards were detected in fractions after collection visually (Blue Dextran) or by the phenol-sulphuric colorimetric assay (glucose). The supernatant residue was dissolved in a minimal volume and loaded onto the column, eluting with MilliQ-purified water at a flow rate of 6 ml/h and collecting 0.5 ml fractions. Aliquots were taken from each fraction and analyzed for carbohydrate content by the phenol-sulphuric colorimetric assay and for protein content by the BCA assay; the detection limit for both colorimetric assays was 0.5 μg/ml. Based on the resulting carbohydrate elution profile, fractions representing every 8 ml were pooled and lyophilized to give 5 total fractions spanning the void volume and included volume. Each pooled fraction was dissolved in water and 150 μl aliquots were bioassayed. The precipitated fraction was chromatographed in an identical manner and fractions were collected, assayed for carbohydrate content, and pooled to give five total fractions. Each pooled fraction was dissolved in water and 75 μl aliquots were bioassayed. A positive control using live *Vaucheria longicaulis* induced 84 ± 10% metamorphosis, while a negative control using FSW gave 4 ± 4% background metamorphosis.

Sequential extraction of Vaucheria longicaulis *with solvents of increasing polarity*

To determine whether macromolecules associated with the algal cell wall were bioactive, *Vaucheria longicaulis* was sequentially extracted with solvents of increasing polarity and harshness to extract molecules of increasing molecular weight. Lyophilized *Vaucheria longicaulis* (500 mg) was homogenized into a fine powder and extracted with 80% aqueous ethanol (50 ml, 7 h, 75°C), cold water (50 ml, 4 d, 20°C), hot water (50 ml, 24 h, 65°C), and 4% sodium hydroxide (50 ml, 24 h, 20°C) (Cleare and Percival, 1972). The ethanol extract was partitioned into a water-soluble fraction and a water-insoluble organic fraction. The cold and hot water extracts were precipitated with ethanol as before to generate supernatant and precipitate fractions for each extract. Aliquots corresponding to 250 μg dry weight were taken from the water-soluble ethanol extract and from the cold and hot water supernatant and precipitate fractions and were assayed directly for bioactivity. An aliquot of the

organic-soluble material from the ethanol extract was dissolved in methanol, added to a dry culture dish, the solvent evaporated, and 4 ml of FSW added prior to the bioassay.

The 4% NaOH extract was exhaustively dialyzed against MilliQ-purified water and lyophilized, giving a dry material (44 mg) that was completely insoluble in water but dissolved readily in 7 M urea. The S-200 Sephacryl column was equilibrated in 7 M urea and calibrated for V_o and V_t as before. A portion of the 4% NaOH extract was dissolved in a minimal volume of 7 M urea and loaded onto the S-200 column. The sample was chromatographed and fractions were collected and assayed exactly as before, except the column was eluted with 7 M urea. Fractions comprising the high molecular weight carbohydrate peak were pooled and dialyzed exhaustively against water using 10,000 molecular weight cutoff dialysis tubing. The dialysate was reduced to a volume of 1 ml on a rotary evaporator and 100 μl aliquots were bioassayed.

Treatment of BVE with proteinase K, sodium periodate, and mild acid hydrolysis

Chemical and enzymatic treatments were performed to determine the biochemical nature of the settlement cue. A solution of sodium periodate (0.37 M, 100 μl) was added to 1.0 ml of BVE, and the solution was incubated at 4°C in the dark (Hassid and Abraham, 1957). The reaction was quenched after 24 h by the addition of excess glycerol (20 μl). As a control, 1.0 ml of BVE was incubated at 4°C in the dark for 24 h, after which excess glycerol (20 μl) was added followed immediately by periodate as in the treated sample. Both samples were incubated for 1 h to allow the consumption of excess periodate, and were then dialyzed exhaustively against deionized water for one week. Both treatment and control samples were lyophilized, dissolved in 300 μl FSW, and 100 μl aliquots used as replicate treatments in the larval settlement bioassay.

Proteinase K (600 μg) was added to a sample of BVE (300 μl) which had been adjusted to pH 7.8 and incubated at 50°C for 24 h. The proteinase was then inactivated by heating at 100°C for 15 min. A control was done by adding proteinase to BVE immediately prior to heating at 100°C for 15 min. Samples were split into three replicate 100 μl aliquots and tested in the larval bioassay. A mild acid hydrolysis was performed by adding concentrated TFA (1.5 μl) to BVE (400 μl) to achieve a final concentration of 0.1 M TFA. The solution was heated at 100°C for 75 min (Lahaye and Ray, 1996) and dried under vacuum to remove TFA. As control for the presence of residual TFA salts, BVE (400 μl) was heated in parallel at 100°C for 15 min, and concentrated TFA was added to BVE immediately prior to drying under vacuum. Samples were dissolved in 300 μl FSW, and 100 μl aliquots used as replicate treatments in the bioassay. Differences between treatment and control samples were compared using an unpaired two-tailed t-test on arcsine-transformed percentages for each of the three treatments, as different quantities of BVE were treated and bioassayed in each case.

Results

Secreted and surface-associated forms of the larval settlement cue

Previous work had demonstrated that *Alderia modesta* larvae metamorphosed specifically in response to living tissue of *Vaucheria longicaulis* (Krug, 1998a). The initial aim of the present study was to determine whether the settlement cue was secreted into seawater by living algae, and whether dead or homogenized algal tissue could induce settlement. Water previously conditioned by the presence of *V. longicaulis* was as active in promoting metamorphosis as was the living algae (Fig. 1A, and results of a 1-way ANOVA: df = 4, 22; $F = 32.73$; $P < 0.0001$). The

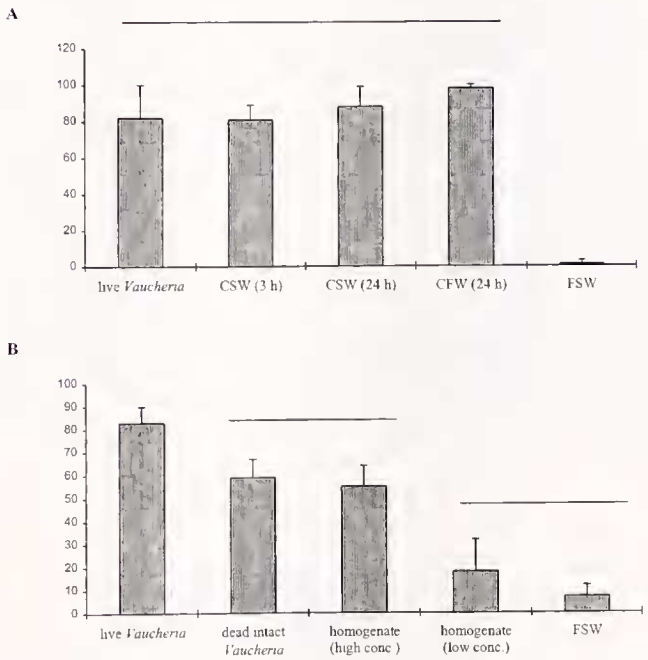

Figure 1. Induction of larval metamorphosis by live *Vaucheria longicaulis*, dead tissue, and conditioned water. Percentages of larval metamorphosis are given as means + SD (n = 3); arcsine-transformed percentages were compared with a 1-way ANOVA, with a post-hoc Scheffé test for unplanned comparisons. Live *V. longicaulis* tissue was used as a positive control and filtered sea water (FSW) as a negative control A. Secretion of larval settlement cue by living *V. longicaulis*. Means are percentages of metamorphosis induced by exposure to *Vaucheria*-conditioned seawater (CSW) or conditioned fresh water (CFW). Duration of conditioning process is given in parentheses. Means not joined by a horizontal line differed significantly ($P < 0.001$). B. Inductive effect of dead or homogenized *V. longicaulis*. Previously frozen and thawed *Vaucheria* tissue, or aliquots of homogenized algal tissue, were assayed for inductive effect. Means not joined by a horizontal line differed significantly ($P < 0.05$).

conditioning process occurred rapidly in the laboratory, such that water conditioned for 3 h induced the same level of metamorphosis as water conditioned for 24 h. Fresh water was also conditioned by the presence of *V. longicaulis* (Fig. 1A). There was no statistical difference between the level of metamorphosis induced by the living algae and any of the conditioned water treatments, all of which differed significantly from the seawater-only control (Scheffé test, $P < 0.001$).

Vaucheria longicaulis tissue that was frozen and thawed induced significant larval metamorphosis, indicating that the algae does not have to be alive to trigger settlement (Fig. 1B, and results of a 1-way ANOVA: df = 4, 16; $F = 61.55$; $P < 0.0001$). Homogenates of algal tissue were also active, confirming that *V. longicaulis* tissue does not have to be alive or intact to induce metamorphosis (Fig. 1B). Significantly higher levels of metamorphosis were induced by frozen *V. longicaulis* and the higher concentration of tissue homogenate than by the negative control (Scheffé test, $P < 0.05$). The lower concentration of homogenate did not induce significantly more metamorphosis than the negative control, indicating that the larvae may be dose-responsive to preparations of the cue; dilution experiments with conditioned seawater support this conclusion (data not shown).

When *Vaucheria longicaulis* was extracted with boiling water, the resulting aqueous extract was as active as positive controls when assayed at an 80-fold dilution (Fig. 2, and results of a 1-way ANOVA: df = 8, 18; $F = 20.45$; $P < 0.0001$). Conditioned seawater had no effect at such a dilution, indicating that boiling water extracted the settlement cue more efficiently than did the conditioning process. When the *V. longicaulis* tissue was re-extracted with boiling water for a second time, the resulting extract induced a low level of metamorphosis, but not significantly more than the negative control when assayed at an 80-fold dilution (Fig. 2). Four further extractions with boiling water yielded extracts that contained no appreciable bioactivity, even when assayed at higher concentrations. These data indicate that all of the measurable bioactivity was extracted from *V. longicaulis* in the first two boiling water treatments. The insoluble residue remaining after six sequential extractions had thus been exhaustively extracted. However, larvae exposed to this residue metamorphosed at a level comparable to those exposed to living *V. longicaulis* (Fig. 2). Significant bioactivity thus remained associated with the *Vaucheria* cell wall residue after all the soluble settlement cue had been extracted.

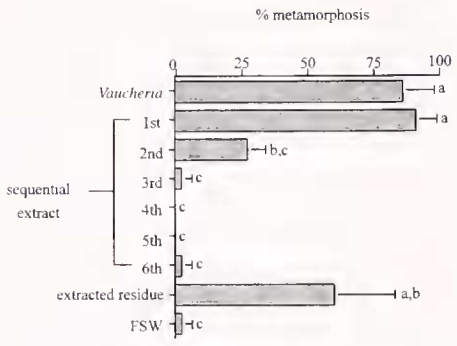

Figure 2. Serial extraction of *Vaucheria longicaulis* with boiling water. Means + SD (n = 3) are percentages of larval metamorphosis induced by aliquots of 6 sequential boiling water extracts, tested at an 80-fold dilution, along with the fully extracted algal residue. Live *V. longicaulis* was used as a positive control, and FSW as a negative control. Means not identified with the same letter differed significantly ($P < 0.05$, 1-way ANOVA with a post-hoc Scheffé comparison).

High and low molecular weight forms of the soluble settlement cue

Boiled *Vaucheria* extract (BVE) was fractionated by ethanol precipitation into a supernatant and precipitate, each of which was diluted back up to the starting volume of BVE for comparison. Biochemical analysis revealed that the carbohydrate content of BVE partitioned equally between the precipitate and supernatant, while the majority of the protein in the crude BVE went into the ethanol precipitate (Table I). There was no significant difference between the bioactivity in 100 μl of precipitate, supernatant, and BVE (1-way ANOVA, $P > 0.3$), although the supernatant consistently displayed slightly lower activity at several concentrations tested.

Both the ethanol precipitate and supernatant were further fractionated by gel filtration chromatography on a Sephacryl S-200 column. When column fractions were assayed for carbohydrate content, contrasting elution profiles were obtained for the two samples (Fig. 3). All detectable carbohydrate from the supernatant fraction eluted in the included volume of the column, indicating a molecular weight of <2,000 Da. In contrast, when the precipitate was chromatographed, all detectable carbohydrate eluted as one peak in the void volume, indicating molecules of >100,000 Da molecular weight. When fractions were pooled and bioassayed, there was significant variation in the bioactivity of different fractions (Fig. 3, and results of a 1-way ANOVA: df = 11, 24; $F = 17.33$; $P < 0.0001$). For the precipitate, a high level of metamorphosis (54 ± 23% SD) was induced by the pooled fractions containing the high molecular weight carbohydrate peak, and a lower level was induced by the adjacent fraction containing the trailing edge of the peak. The level of metamorphosis induced by the high molecular weight peak was not statistically different from that induced by the positive control (Scheffé test, $P = 0.20$) but was significantly higher than the negative control (Scheffé test, $P < 0.05$). No bioactivity significantly higher than the negative control (4 ± 4%) was detected in the low molecular weight fractions from the ethanol precipitate. The bioactivity profile of the ethanol supernatant gave the op-

Table I

Comparative dry weight, protein content, carbohydrate content, and bioactivity (±SD) of 100 µl aliquots of a standard solution of boiled Vaucheria *extract (BVE) and the precipitate and supernatant resulting from ethanol treatment of BVE. The precipitate and supernatant were dissolved in the starting volume of extract and aliquots were removed for chemical assays (n = 2) and bioassays (n = 3)*

Sample	Dry Weight (µg)	Carbohydrate (µg)	Protein (µg)	Bioactivity (%)
BVE	270 ± 10	6 ± 1	25 ± 1	82 ± 25
supernatant	110 ± 10	4 ± 1	6 ± 1	49 ± 4
precipitate	140 ± 10	3 ± 1	17 ± 1	77 ± 27

posite result. The low molecular weight fraction of the supernatant, which contained all the carbohydrate, induced a level of metamorphosis that was not significantly different from the high molecular weight carbohydrate peak from the precipitate (Scheffé test, $P = 0.79$). No other fraction from the supernatant induced significant metamorphosis. Bioactivity thus co-eluted with the major carbohydrate peak of both the supernatant and precipitate, although the active peak from the supernatant contained only low molecular weight molecules while that from the precipitate contained molecules of high molecular weight. Identical carbohydrate peak profiles were obtained when samples were chromatographed using 7 M urea as a chaotropic agent to disrupt any potential aggregation of macromolecules, and no major protein peaks were evident for either sample (data not shown).

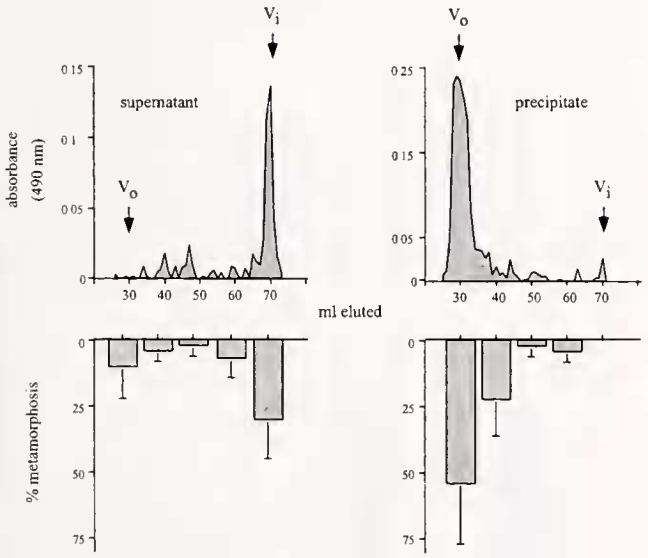

Figure 3. Gel filtration chromatography of the supernatant and precipitate from ethanol precipitation of boiled *Vaucheria* extract (BVE). Fractions were independently chromatographed on a size-calibrated column of Sephacryl S-200 gel eluting with water. Molecules of molecular weight > 100,000 Da elute in the void volume (V_o), while those of <2,000 Da elute in the included volume (V_i). Column fractions (0.5 ml) were assayed for carbohydrate content by the phenol-sulphuric colorimetric assay. Fractions were pooled as indicated, lyophilized, and bioassayed for induction of larval metamorphosis. Percentages of metamorphosis are means + SD (n = 3).

Sequential extraction of Vaucheria longicaulis

Lyophilized *Vaucheria longicaulis* was sequentially extracted with solvents of increasing harshness to determine if bioactivity was persistently associated with molecules of increasing molecular weight and stronger association with the algal cell wall. Aqueous extracts were ethanol precipitated to yield supernatant and precipitate fractions, and all soluble extracts were bioassayed at the same concentration per unit dry weight. The material extracted with 4% NaOH was insoluble in water but dissolved readily in 7 M urea, a chaotropic agent routinely used to solubilize and chromatograph high molecular weight polysaccharides. One major carbohydrate peak was detected in the void volume of the S-200 column when the 4% NaOH extract was chromatographed with 7 M urea as eluant (Fig. 4). This carbohydrate peak was exhaustively dialyzed, and the material which remained in aqueous solution was bioassayed. There was significant variation in the bioactivity of different extracts (Fig. 5, and results of a 1-way ANOVA: df = 8, 39; $F = 4.64$; $P < 0.0005$). The water-soluble partition of an ethanol extract of *V. longicaulis* induced significantly higher levels of metamorphosis than the water-insoluble organic layer and the negative control (Scheffé test, $P < 0.05$), indicating all bioactive molecules are highly polar. Bioactivity above the level of the negative control (8 ± 8%) was found in all water-soluble extracts as well as in the resolubilized 4% NaOH extract, indicating that molecules of increasing mo-

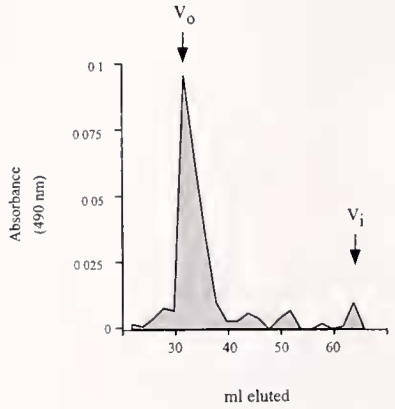

Figure 4. Carbohydrate elution profile of 4% NaOH extract of *Vaucheria longicaulis* powder. Aqueous-insoluble material from the basic extraction was eluted from Sephacryl S-200 gel with the chaotropic agent 7 M urea. Fractions containing the carbohydrate peak eluting in the void volume were pooled, dialyzed, and reduced in volume before being bioassayed.

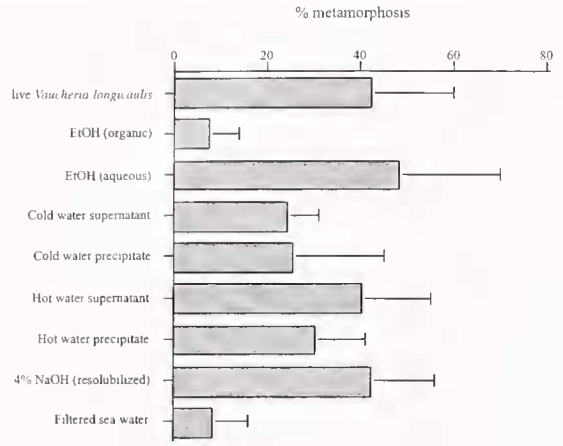

Figure 5. Bioactivity of sequential extracts of *Vaucheria longicaulis*. Lyophilized *Vaucheria* powder was sequentially extracted with aqueous ethanol, cold water, hot water, and 4% NaOH. The ethanol extract was partitioned into aqueous and organic fractions. Cold and hot water extracts were precipitated with ethanol to yield supernatant and precipitate fractions for each. Aliquots of equal dry weight were bioassayed for all water-soluble extracts; the carbohydrate peak from the 4% NaOH extract (see Fig. 4) was dissolved in water prior to the bioassay. Percentages of metamorphosis are means + SD (n = 3).

lecular weight are all active in promoting larval settlement, including those associated with structural elements of the cell wall (Fig. 5).

Chemical and enzymatic treatment of boiled Vaucheria *extract (BVE)*

To determine whether proteinacious components were required for bioactivity, BVE was treated with a high concentration (2 mg/ml) of proteinase K. Proteinase treatment did not decrease the bioactivity of BVE (Fig. 6). Bioactivity was also stable to mild acid hydrolysis using 0.1 M TFA at 100°C. In contrast, treatment with sodium periodate, a chemical agent which cleaves sugar residues, significantly decreased the bioactivity of BVE (Fig. 6).

Discussion

The present study demonstrates that larvae of the ascoglossan *Alderia modesta* metamorphose in response to both secreted and surface-associated forms of the settlement cue derived from the adult host alga, *Vaucheria longicaulis*. Seawater conditioned by the presence of *V. longicaulis* was as active as the algae itself at inducing larval metamorphosis, suggesting the algae secretes one form of the cue into the surrounding seawater. Water-soluble cues derived from the adult prey organism induce larval settlement for other specialist predators. Larvae of the aeolid nudibranch *Phestilla sibogae* metamorphosed in response to water conditioned with the hard coral *Porites compressa* (Hadfield, 1977; Hadfield and Scheuer, 1985). Larvae of the dorid nudibranch *Adalaria proxima* metamorphosed in seawater conditioned by the preferred adult prey, the bryozoan *Electra pilosa* (Lambert and Todd, 1994). However, metamorphosis of *A. proxima* larvae could only be induced by live colonies of *E. pilosa* and not by dead colonies or homogenized extracts (Todd *et al.*, 1991; Lambert and Todd, 1994). In contrast, dead and homogenized *V. longicaulis* tissue induced metamorphosis in *A. modesta*.

Secreted settlement cues are also involved in gregarious settlement of some species. Larvae of the sand dollars *Dendraster excentricus* and *Echinarachinus parma* metamorphosed in response to sand beds and seawater conditioned by the presence of adult conspecifics (Burke, 1984; Pearce and Scheibling, 1990). The most detailed studies on the effects of a secreted chemical settlement cue have focused on the oyster *Crassostrea virginica*. Larvae altered their swimming speed and turning rate in response to small basic peptides secreted by adult conspecifics, significantly increasing settlement in both still and moving water in response to the dissolved cue (Tamburri *et al.*, 1992; Turner *et al.*, 1994; Tamburri *et al.*, 1996). These results demonstrate that soluble chemicals can increase settlement rates by influencing the behavior of larvae still suspended in the water column (Turner *et al.*, 1994). The relative importance of the secretion of the *Vaucheria*-derived cue to the settlement of *Alderia modesta* larvae in the field will require further study. However, the rapidity of the conditioning process suggests that absorbent mats of *V. longicaulis* may become saturated with naturally conditioned water during high tides, which might induce settlement in larvae that enter trapped water parcels.

Extracting *Vaucheria longicaulis* with boiling water not only demonstrated that the chemical cue is stable to prolonged periods of boiling, but that a highly concentrated solution can be prepared in this manner. The diminishing bioactivity of sequential extracts indicates that

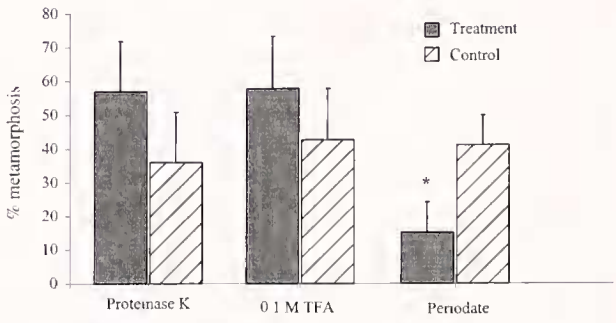

Figure 6. Effects of proteinase K, mild acid hydrolysis, and sodium periodate on bioactivity of boiled *Vaucheria* extract (BVE). Percentages of larval metamorphosis are given as means + SD (n = 3) for each treatment and the corresponding control. Percentages for each treatment and control were arcsine transformed and compared with a two-tailed unpaired t-test (* = significant at $P < 0.05$ level); the t values obtained were 2.08 (proteinase K), 1.20 (mild acid hydrolysis), and −4.46 (sodium periodate).

there is a limited amount of the waterborne cue that can be extracted with boiling water. However, after repeated extractions the residual *Vaucheria* tissue retained significant activity, indicating that a non-extractable form of the settlement cue remains associated with the algal cell wall. This is the first direct demonstration that the same substrate produces both secreted and surface-associated forms of a larval settlement cue, each of which is sufficient to induce metamorphosis.

Polysaccharide chemists routinely employ basic solvents such as NaOH to extract material that remains associated with plant cell walls following hot water extraction (Cleare and Percival, 1972). The water-insoluble material extracted with 4% NaOH contained high molecular weight carbohydrates that, when partially resolubilized in 7 M urea and then dialyzed against water, were active in the larval settlement assay. The bioactivity of this fraction is consistent with the finding that *Vaucheria* tissue exhaustively extracted with boiling water can still induce metamorphosis; together, these experiments define a surface-associated class of molecules which differ in their physical properties (size, solubility) from the water-soluble cue molecules, but share the same bioactivity. An insoluble inducer associated with cell wall polysaccharides of the crustose red alga *Hydrolithon boergesenii* triggered metamorphosis in the coral *Agaricia humilis* (Morse *et al.*, 1988; Morse and Morse, 1991). Larvae of the echinoid *Stronglyocentrotus droebachiensis* metamorphosed in response to live tissue or a homogenate of several species of coralline red algae, but the algae did not release soluble inducers into seawater (Pearce and Scheibling, 1990).

The active material in the water-soluble extracts was further divided into discrete molecular weight classes by ethanol precipitation. Chromatography revealed that the carbohydrates in the BVE precipitate were exclusively of high molecular weight, while those in the supernatant were all of low molecular weight; in both cases, the bioactivity co-eluted with the major carbohydrate peak. Studies of larval settlement inducers for other opisthobranchs have used ultrafiltration to show that the bioactive molecules are less than 1,000 Da in size (Hadfield and Pennington, 1990; Gibson and Chia, 1994; Lambert *et al.*, 1997). Distinct size-classes of water soluble settlement cue molecules have not been previously reported from other study systems.

The bioactive settlement cue molecules co-eluted with the carbohydrate peak in each extract, were stable to boiling and mild acid or base treatment, and some were firmly associated with the algal cell wall. These results suggested that the molecules were either composed of, or tightly associated with, algal carbohydrates. Proteinase K treatment did not diminish the activity of algal extracts, but bioactivity was significantly reduced by treatment with sodium periodate. Periodate reacts with monosaccharide units of polysaccharides, oxidizing consecutive hydroxyl groups to aldehydes and cleaving sugar residues having three consecutive hydroxyl groups to produce formic acid (Hassid and Abraham, 1957). Taken together, the data strongly suggest that the larvae of *Alderia modesta* metamorphose in response to a structural feature of the polysaccharides produced by *Vaucheria longicaulis*. This would account for the bioactivity of molecules of differing molecular weight, since small oligosaccharides can contain the same distinctive glycosidic linkages as are found in the full-length polymer. Consistent with this hypothesis, the activity of *Vaucheria* extract was not diminished by a mild acid hydrolysis using 0.1 M TFA; the same conditions have been used to fragment matrix polysaccharides of green algae into smaller oligosaccharides representative of the repeating unit (Lahaye and Ray, 1996).

Recognition of carbohydrates by larval lectins has been implicated in settlement induction for several taxonomically diverse marine invertebrates (Kirchman *et al.*, 1982; Maki and Mitchell, 1985; Bahamondes-Rojas and Dherbomez, 1990; Bonar *et al.*, 1990; Morse and Morse, 1991). Metamorphosis of barnacle larvae in response to glycoproteins is abolished when the oligosaccharide chains of the proteins are bound by lectins and thus rendered inaccessible to larval receptors (Matsumura *et al.*, 1998). The present study strongly indicates a carbohydrate is the settlement cue for *Alderia modesta*, but definitive proof will require the isolation of a pure oligosaccharide that induces metamorphosis. Preliminary results indicate that inductive fragments are anionic and contain uncommon sugar residues including glucuronic and galacturonic acid, rhamnose, and xylose, which are not recognized by most available enzymes and lectins (Krug, 1998a). A direct chemical analysis of the structural features of the polysaccharides of *Vaucheria longicaulis* and their bioactivity is currently underway. However, bioactivity is clearly associated with algal polysaccharides, both soluble and insoluble, making *A. modesta* an ideal experimental organism for dissecting the roles of waterborne versus surface-associated cues in the larval settlement process.

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